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Developmental Expression of Secretory β-1,4-endoglucanases in the Subventral Esophageal Glands of *Heterodera glycines*

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Two β-1,4-endoglucanases (EGases), *Hg-eng-1* and *Hg-eng-2*, were recently cloned from the soybean cyst nematode, *Heterodera glycines*, and their expression was shown in the subventral esophageal glands of hatched second-stage juveniles (J2). We examined the expression of these EGases in the subventral glands of all post-embryonic life stages of *H. glycines* by in situ hybridization and immunolocalization. The first detectable accumulation of EGase mRNAs occurred in the subventral glands of unhatched J2. EGase transcripts remained detectable in J2 after hatching and during subsequent root invasion. However, in late parasitic J2 and third-stage juveniles (J3), the percentage of individuals that showed EGase transcripts decreased. In female fourth-stage juveniles and adult females, EGase transcripts were no longer detected in the subventral glands. EGase hybridization signal reappeared in unhatched males coiled within the J3 cuticle, and transcripts were also present in the subventral glands of migratory adult males. Immunofluorescence labeling showed that EGase translation products are most abundantly present in the subventral glands of preparasitic J2, migratory parasitic J2, and adult males. The presence of EGases predominantly in the migratory stages suggests that the enzymes are used by the nematodes to soften the walls of root cells during penetration and intracellular migration.

Additional keyword: cellulase.

Cyst nematodes (*Heterodera* and *Globodera* spp.) are highly specialized endoparasites that obtain nutrients from their host plant through the formation of syncytial feeding cells within the roots (Jones 1981; Melillo et al. 1990). The post-embryonic development of cyst nematodes is dependent upon parasitism for completion, and is characterized by four successive juvenile stages, which undergo molts to reach adulthood (Raski 1950). The molt to the preparasitic (or infective) second-stage juvenile (J2) occurs within the egg (Hagemeyer 1951). The preparasitic J2 hatches from the egg in the soil and penetrates plant roots. Within the root, the now parasitic J2 migrates intracellularly through the cortex to the vascular cylinder. Here, the J2 inserts its stylet, a hollow mouth spear, into a selected parenchyma cell and induces its transformation into an initial syncytial feeding cell (Wyss and Grundler 1992). The now feeding J2 swells and changes into a sedentary parasite as adjacent root parenchyma cells are incorporated into the growing syncytium by cell wall dissolution (Jones 1981; Wyss and Grundler 1992). Juveniles that develop into females continue to feed from the syncytium throughout all successive life stages, and their bodies swell to become lemon-shaped (*Heterodera* spp.) or round (*Globodera* spp.) by the time they reach sexual maturity. The posterior part of the growing female eventually protrudes from the root. After fertilization, the adult female fills with eggs, and her body wall hardens to form a protective cyst to encase the eggs. Nematodes that develop into males stop feeding after the third juvenile (J3) stage. Within the shed J3 cuticle, the fourth-stage (J4) male undergoes a maturation to the adult stage, during which it becomes vermiform again and regains the ability to migrate (Günther 1972; Raski 1950). Following eclosion from the J3 and J4 cuticles, the adult male exits the root to mate with the sedentary females.

Stylet secretions originating from one dorsal and two subventral esophageal gland cells of endoparasitic nematodes are thought to play an important role in the interaction of these parasites with their host plants (Hussey 1989a; Williamson and Hussey 1996). These secretions are packaged in secretory granules, which are transported into a narrow, anterior gland cell extension (Endo 1984). Secretions are released into the esophagus at the end of these extensions, and then moved forward to be released through the stylet. Several initiatives have been undertaken in the past decade to identify esophageal gland secretions by raising monoclonal antibodies (MAbs) to gland antigens (Atkinson et al. 1988; Davis et al. 1992; de Boer et al. 1996a, 1996b; Goverse et al. 1994; Hussey 1989b; Ray et al. 1994). Such MAbs have been used to identify and characterize secretory proteins from the subventral glands of preparasitic J2 of *Globodera rostochiensis* (de Boer et al. 1996b; Smant et al. 1997). The amino acid sequence of the affinity-purified antigen of one of these MAbs

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was used to clone two β-1,4-endoglucanase (EGase) cDNAs from both *G. rostochiensis* and *H. glycines* that were expressed in the subventral glands of preparasitic J2 (Smant et al. 1998; Yan et al. 1998). In *H. glycines*, the two genes were named *Hg-eng-1* and *Hg-eng-2*. While *Hg-eng-1* encodes an EGase that has a catalytic domain linked to a cellulose-binding domain, *Hg-eng-2* encodes an EGase that only contains a catalytic domain.

A recent report has documented the in planta secretion of HG-ENG-2 cellulase by J2 of *H. glycines* 24 h after inoculation of soybean roots (Wang et al. 1999). However, more detailed developmental expression data are required to assess the potential functions of HG-ENG-1 and HG-ENG-2 cellulases throughout the life cycle of *H. glycines*. In this paper we present an in depth microscopic expression analysis of cellulase genes on the level of mRNA and protein accumulation within the subventral glands of all post-embryonic life stages of *H. glycines*. As such, this paper represents the most detailed expression analysis of any plant-parasitic nematode gene to date.

RESULTS

In situ hybridization to EGase mRNAs.

For the detection of EGase transcripts, successive life stages of *H. glycines* were hybridized with two digoxigenin-labeled antisense RNA probes that were transcribed from cDNA of *Hg-eng-1* and *Hg-eng-2*, respectively. The *Hg-eng-1* probe corresponded to the cellulose-binding domain unique to *Hg-eng-1*. The *Hg-eng-2* probe corresponded to a 176-bp region in the catalytic domain of *Hg-eng-2*. In spot blot hybridizations, the *Hg-eng-2* probe showed negligible binding to *Hg-eng-1* RNA (data not shown), demonstrating that this probe could be used to detect *Hg-eng-2* transcripts in situ. The hybridized probes were visualized with direct alkaline phosphatase immunostaining.

To determine the time point in the life cycle at which EGase transcripts become detectable, eggs were isolated from mature females and allowed to develop for 2 more days in $ZnSO_4$ hatching solution at 25°C. These eggs displayed a continuum of developmental stages, ranging from embryonic tissue to fully developed J2. Upon hybridization to the eggs, both antisense *Hg-eng* probes produced a strong staining signal within the subventral glands of fully developed J2 (Fig. 1A). A proportion of the J2 did not hybridize with the EGase riboprobes. Closer examination of these juveniles showed that their subventral glands were not yet filled with secretory granules, indicating that these J2 were not yet ready to hatch (Perry et al. 1989). No EGase transcripts were detected if eggs were not incubated on hatching solution and used immediately for in situ analysis. This result further indicated that the EGase transcripts only become detectable when the J2 have developed to the stage at which they are close to hatching.

With both antisense probes, a strong hybridization signal remained present in the subventral glands of J2 after they had hatched from the egg and during their migration within the root (Fig. 1B, C). The subventral gland extensions usually remained unstained in the hatched preparasitic J2 (Fig. 1B) but strongly stained after the J2 had invaded the host plant (Fig. 1C).

After initiating a feeding site and becoming sedentary, the now enlarged parasitic J2 continued to exhibit both EGase transcripts in their subventral glands (Fig. 1D, E). In the sedentary J2 stage, the gland extensions were no longer stained (Fig. 1D) and the size of the subventral glands decreased (Fig. 1E). In the J2 stage corresponding to Figure 1D, the genital primordium had already progressed to the six-cell stage. Transcripts of both genes also were detected in the subventral glands of J3 (Fig. 1F). Although both antisense EGase riboprobes hybridized to the subventral glands in nearly 100% of the preparasitic and migratory parasitic J2 specimens examined, the percentage of EGase-positive individuals decreased in the sedentary J2 and J3 stages. In late parasitic J2, the percentages of individuals positive with antisense *Hg-eng-1* or *Hg-eng-2* probe were 27% (n = 22) and 25% (n = 8), respectively, and in the J3 stage these values were 16% (n = 13) and 27% (n = 22), respectively. Of the J3 individuals that tested positive for EGase mRNA, those probed with *Hg-eng-2* invariably produced a strong hybridization signal (Fig. 1F), whereas those probed with *Hg-eng-1* exhibited a reduced intensity of the alkaline phosphatase stain, indicating decreased amounts of transcripts in the glands.

EGase mRNAs were not detected in the subventral glands of female J4, adult females, or male J4 at the beginning of their stretching phase (see Figure 12C in Günther 1972). However, with both antisense probes, strong hybridization signals returned in the subventral glands of fully stretched males that were still coiled within the J3 cuticle and that were close to hatching (Fig. 1G). It was uncertain whether at the stage shown in Figure 1G all EGase-positive males had already molted to adults, but in some stained individuals a shed J4 cuticle was clearly visible. Transcripts of both genes remained present in adult males that were migrating within the root tissue (Fig. 1H).

Control hybridizations with sense *Hg-eng-2* probe showed no binding to preparasitic J2 (de Boer et al. 1998), hatching eggs, or parasitic J2 of *H. glycines*. Sense *Hg-eng-1* probe was tested on *H. glycines* hatching eggs and preparasitic J2, and showed no binding.

Immunolocalization of EGase proteins.

To monitor the accumulation of EGase proteins within the subventral glands, life stages of *H. glycines* were probed with antisera that were raised against fusion proteins of HG-ENG-1 and HG-ENG-2 (Smant et al. 1998; Wang et al. 1999). Female J4 and adult females were probed with an antiserum that reacted with both HG-ENG-1 and HG-ENG-2. The other life stages were probed with two antisera that were specific to HG-ENG-1 and HG-ENG-2, respectively. Antiserum binding was examined with indirect immunofluorescence microscopy.

The first detectable appearance of EGase proteins was in unhatched J2 within the egg (Fig. 2A, B): both antisera produced a strong, even fluorescence signal throughout the subventral gland cells, including their extensions. Strong signals of antiserum binding were observed for both EGases in hatched preparasitic J2 and in migratory parasitic J2 (Fig. 2C, D). Although, in early sedentary J2, abundant quantities of EGase occasionally could be observed (Fig. 2E), most specimens in this developmental stage showed a marked decrease of fluorescence signal, indicating lower amounts of EGase present within the subventral gland cells (Fig. 2F). In the late sedentary J2 stage, only low quantities of both EGases remained detectable in the subventral glands (Fig.

2G, H). In J3, HG-ENG-1 was no longer detected, but low concentrations of HG-ENG-2 remained present within the subventral gland cells and extensions (Fig. 2I, J). In female J4 and adult females, no EGases were detected in the subventral glands. In fully stretched males that were still coiled within their J3 cuticles, generally low levels of both EGases could be detected in the subventral glands and their extensions (Fig. 2K). Migratory adult males showed a strong labeling of the subventral glands with the HG-ENG-1 and HG-ENG-2 antisera (Fig. 2L).

DISCUSSION

The developmental expression pattern of EGases in *H. glycines* suggests that their primary function is associated with root penetration and migration through root tissues by migratory J2 and emigrating males. Our observations provide a clearer support for an earlier hypothesis that EGases are secreted by migratory cyst nematode stages to soften the cell walls, thereby facilitating intracellular migration through the root cells (Smant et al. 1998). This notion is also supported by

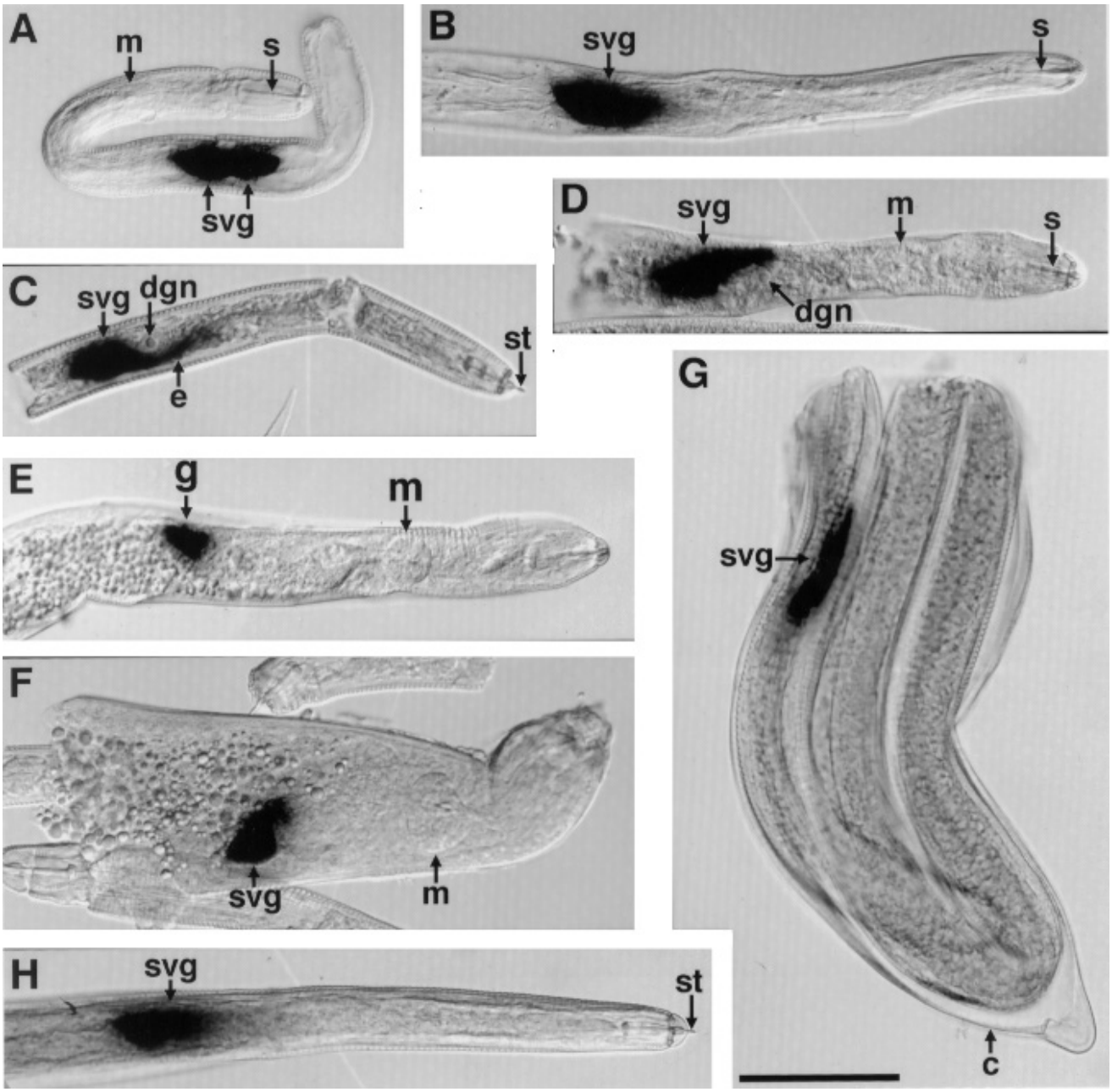

Fig. 1. In situ hybridizations of β-1,4-endoglucanase (EGase) riboprobes to the subventral glands (svg) in life stages of *Heterodera glycines*. Sites of in situ hybridization are stained dark. **A,** Reaction of *Hg-eng-1* probe with an unhatched, preparasitic J2. **B,** *Hg-eng-2* probe with hatched, preparasitic J2. **C,** Reaction of *Hg-eng-1* probe with a migratory parasitic J2, showing presence of transcripts also in the subventral gland extensions (e). **D,** *Hg-eng-2* probe with early sedentary parasitic J2. **E,** *Hg-eng-2* probe with a late sedentary parasitic J2. **F,** *Hg-eng-2* probe with a third-stage juvenile (J3). **G,** *Hg-eng-2* probe with an unhatched male, folded within the shed J3 cuticle (c). **H,** *Hg-eng-1* probe with a migratory adult male. m = metacorpus, dgn = dorsal gland nucleolus, s = stylet, st = stylet tip. Scale bar: 50 µm.

preliminary experiments in which live preparasitic J2 and adult males of *H. glycines*, embedded in carboxymethyl cellulose agarose, released a cellulolytic activity (data not shown), and by in situ localization of EGase protein in the migration path of *H. glycines* early parasitic J2 (Wang et al. 1999). This latter study also indicated that J2 may selectively secrete the two EGases during early stages of parasitism. Therefore, a finer analysis of the temporal secretion and activities of HG-ENG-1 and -2 during parasitism will be necessary to elucidate the roles of both cellulases.

Although EGase transcripts and translation products remained detectable in late parasitic J2 and in J3, it is unclear

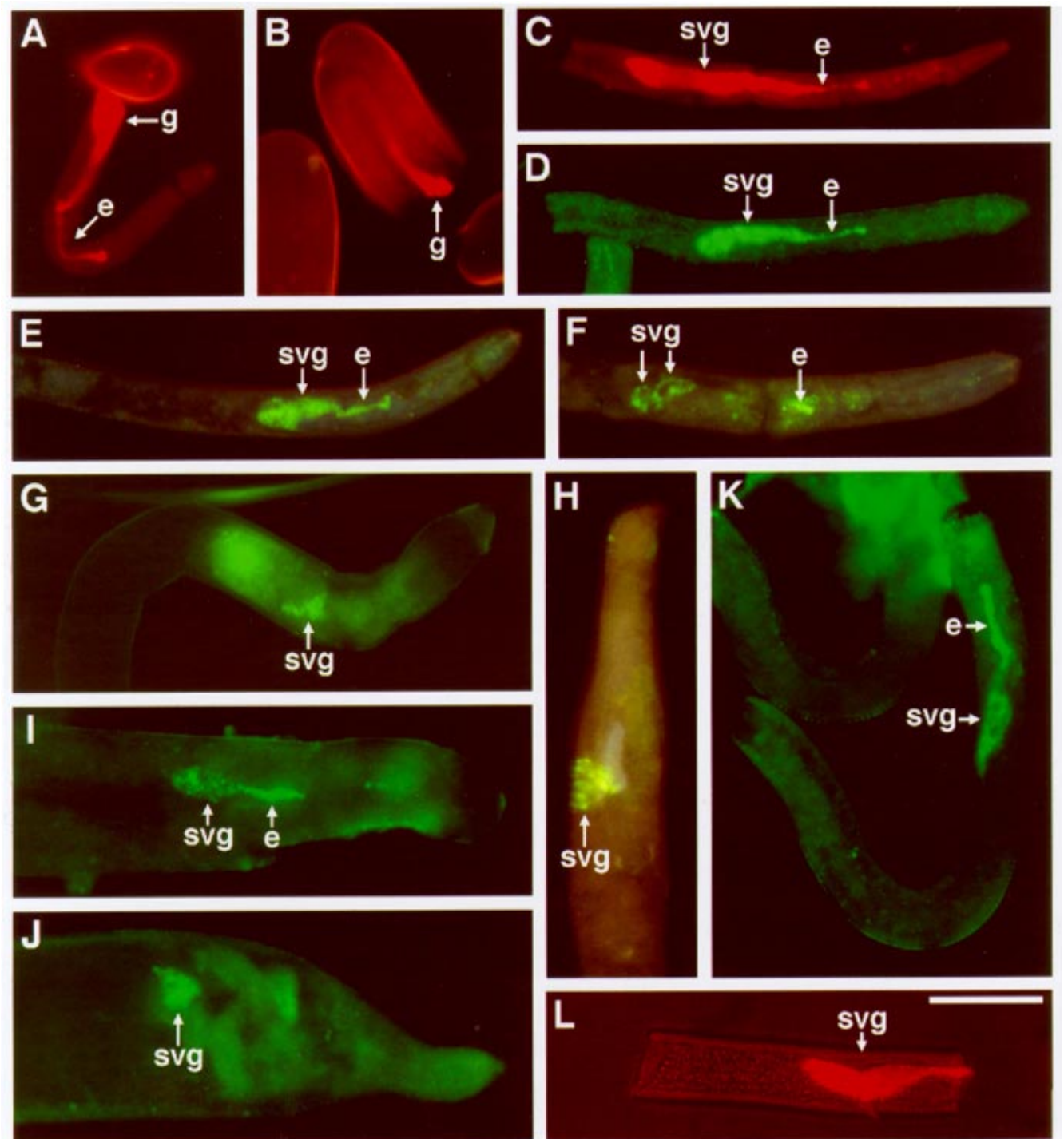

Fig. 2. Localization of the β-1,4-endoglucanase (EGase) translation products HG-ENG-1 and HG-ENG-2 in the subventral glands (svg) and their extensions (e) of life stages of *Heterodera glycines*, with specific antisera. **A** and **B,** Localization of HG-ENG-1 in unhatched, preparasitic J2. **C,** HG-ENG-1 in hatched, preparasitic J2. **D,** HG-ENG-1 in migratory parasitic J2. **E** and **F,** HG-ENG-1 in early sedentary J2. **G,** HG-ENG-2 in late sedentary J2. **H,** HG-ENG-1 in late sedentary J2. **I,** HG-ENG-2 in an early J3. **J,** HG-ENG-2 in a late J3. **K,** Detection of HG-ENG-1 in a body section of an unhatched male. **L,** HG-ENG-1 in a section of a migratory adult male. Scale bar: 50 µm.

whether the EGases have a true function or are purely vestigial in these sedentary stages. It is conceivable that cyst nematode EGases may have a function in the early phases of syncytium formation, when cell walls are partially dissolved. However, it has been postulated that EGases of plant origin are involved in syncytial cell wall breakdown (Jones 1981). Detailed analyses of cyst nematode-infected plant sections for the presence of plant and nematode EGases are currently being conducted to address this issue.

Sedentary J2 showed a substantial decrease in EGase concentrations within the glands, compared with the preceding life stages. Whether this reduction in protein concentration was paralleled by a decrease in mRNA abundance remains unclear, because the alkaline phosphatase hybridization signal was saturated in this stage. The exact sensitivities of our immunodetection and in situ hybridization methods are not known. However, our alkaline phosphatase staining reaction can produce a range of color intensities within the nematodes, and the dark staining of the subventral glands by the EGase probes shows that the concentration of transcripts is far above detection level. Alkaline phosphatase staining is sensitive enough to label centromeres in chromosome spreads (Speel et al. 1994). Our observed decrease in EGase protein labeling in sedentary J2 coincides with a switch to the production of a smaller type of secretory granules by the subventral gland cells (Endo 1993). This correlation indicates that our indirect immunofluorescence labeling protocol effectively monitored cytological changes occurring within the gland cells.

In *G. rostochiensis*, microscopic localization studies of EGase gene products have been limited to hatched preparasitic J2 and parasitic J2, showing presence of EGase proteins within the subventral glands and the esophageal lumen, respectively (de Boer et al. 1996b; Smant et al. 1997, 1998). Immunoblot analysis of protein extracts from *G. rostochiensis* life stages showed that EGases svp39 (GR-ENG-2) and svp49 (GR-ENG-1) are abundantly present in preparasitic J2 and parasitic J2, but undetectable in later parasitic stages. In males only GR-ENG-1 was detected in minor quantities (Smant et al. 1997). We have found that *H. glycines* males express two different EGases in their subventral glands, and that their level of expression is comparable to that of infective J2. Under the assumption that males of *G. rostochiensis* also express their cellulase in the subventral glands, these data may indicate that males of *H. glycines* are more dependent upon EGase secretion for their migration through root tissue than males of *G. rostochiensis*.

Extensive screening of an *H. glycines* genomic library produced additional EGase genes. However, no *H. glycines* EGase gene with a predicted translation product that is different from HG-ENG-1 or HG-ENG-2 has been identified (E. L. Davis, *unpublished data*). This result supports earlier immunoblot and enzyme activity observations that indicate the presence of only ENG-1 and ENG-2 types of cellulase in total protein extracts of *H. glycines* (Smant et al. 1998; Wang et al. 1999). These data furthermore indicate that our *Hg-eng-1* and *Hg-eng-2* riboprobes may have hybridized in situ to transcripts originating from multiple EGase genes of the *eng-1* and *eng-2* type, respectively.

The present paper for the first time used in situ hybridization to localize plant-parasitic nematode gene transcripts throughout development. Our results have shown that expression of EGase gene products within the subventral glands of *H. glycines* has both a temporal and a sex-dependent regulation. In view of the large changes in protein expression that occur among cyst nematode developmental stages (de Boer et al. 1992), we expect that more nematode genes involved in the plant-nematode interaction will show complex expression patterns. Similar findings have been inferred from studies of developmental expression of esophageal gland antigens with MAbs (Atkinson and Harris 1989; Davis et al. 1994). With the in situ mRNA hybridization and polyclonal immunolocalization procedures used here, the expression of such parasitism genes can be analyzed at the transcriptional and translational levels in great detail throughout the nematode life cycle.

MATERIALS AND METHODS

Nematodes.

Heterodera glycines inbred line OP50 (Dong and Opperman 1997) was propagated on soybean cv. Corsoy 79 in a greenhouse. To isolate parasitic juveniles and adult life stages of *H. glycines*, 2-week-old soybean plants were inoculated with preparasitic J2. Infected roots were harvested 8, 11, 16, and 18 days after inoculation for the collection of parasitic J2 and J3, early male J4 and female J4, mature males within the J3 cuticle, and adults, respectively. The roots were homogenized for 30 s at medium speed in a kitchen blender (de Boer et al. 1992). In case the nematodes were to be used for in situ hybridization, the root homogenate was immediately fixed for 1 h at 22°C in 2% paraformaldehyde in M9 buffer (42.3 mM Na_2HPO_4, 22 mM KH_2PO_4, 85.6 mM NaCl, 1 mM $MgSO_4$), to reduce the potential degradation of nematode mRNAs. The root homogenate was washed through sieves with pore sizes of, successively, 850, 250, and 25 µm. Nematodes were purified from the 25-µm root fraction by sucrose centrifugation (de Boer et al. 1996a). The root balls used for isolating the *H. glycines* life stages were washed thoroughly before homogenization. This procedure ensured that these nematode samples were not contaminated by preparasitic J2 or emigrated males adhering to the root surface. Eggs of *H. glycines* were incubated on a sieve (25 µm pore size) in 3 mM $ZnSO_4$ at 25°C until J2 began to hatch. Both the eggs and the hatched preparasitic J2 were used for experiments.

Riboprobes.

The sequences representing nucleotides 1158 to 1615 of the *Hg-eng-1* cDNA (GenBank accession number AF006052) and nucleotides 273 to 447 of the *Hg-eng-2* cDNA (GenBank accession number AF006053) were subcloned into the modified pBlueScript II transcription vector pBST (Hermsmeier et al. 1998), between the *Bam*HI and *Eco*RI cloning sites, and at the *Eco*RI cloning site, respectively. Antisense and sense riboprobes were synthesized in vitro from these subcloned cDNA fragments by run-off transcription with T3 and T7 RNA polymerase, respectively (Kessler 1995). The riboprobes were labeled by adding digoxigenin-11-UTP (Boehringer Mannheim, Indianapolis, IN) to the transcription reaction. The direction of (anti)sense transcription was verified by nucleotide sequencing of the template cDNAs (DNA Sequencing and Synthesis Facility, Iowa State University).

The *Hg-eng-2* riboprobe has a 77% sequence identity with the nucleotide 263 to 437 region of the catalytic domain of the

Hg-eng-1 cDNA. Therefore, the specificity of the digoxigenin-labeled *Hg-eng-2* antisense riboprobe was verified in a spot blot assay, in which the probe was hybridized to unlabeled sense RNA transcribed from the nearly full-length (nucleotides 126 to 1615) *Hg-eng-1* cDNA. Hybridization and further processing of the spot blot was done according to the procedure used for in situ hybridization of nematodes.

In situ hybridization.

In situ hybridizations with the *Hg-eng-1* and *Hg-eng-2* riboprobes to post-embryonic life stages of *H. glycines* were performed essentially as described previously for J2 (de Boer et al. 1998). Briefly, fixed life stages were cut into two to five sections and these sections were permeabilized with proteinase K, methanol, and acetone. The nematode sections were hybridized at 55°C with riboprobe, and labeled with an alkaline phosphatase-conjugated antibody to digoxigenin. Bound riboprobe was detected by overnight staining with nitroblue tetrazolium and bromo-chloro-indolyl phosphate at 5°C. Modifications of the in situ protocol for use with other life stages mainly involved the fixation step in 2% paraformaldehyde in M9 buffer. Eggs were fixed for 5 or 6 days at 5°C, with the addition of 10% sucrose to the fixative solution (Jones et al. 1993). Parasitic J2 and J3 were fixed for 1 day at 22°C. Males were fixed for 1 day at 22°C or for 2 days at 5°C. J4 and adult females were fixed for 1 day at 22°C, followed by 1 day at 5°C. Fixed J3, J4, and adults were placed in a small glass petri dish filled with 0.2% paraformaldehyde in 0.1× M9 buffer. These nematode stages then were cut individually into anterior and posterior sections with a small piece of razor blade attached to a plastic pipette tip. The other fixed life stages were cut into sections in bulk with a vibrating razor blade (de Boer et al. 1998). With males, the proteinase K treatment was shortened from 22 to 10 min, to reduce background staining.

Immunofluorescence labeling.

Immunofluorescence labeling of *H. glycines* life stages with antisera to *H. glycines* EGases was performed essentially as described for *H. glycines* preparasitic J2 and females (Goverse et al. 1994). Female J4 and adult females were probed with a rabbit antiserum that reacted with both HG-ENG-1 and HG-ENG-2 on a Western blot (immunoblot) of preparasitic J2. The other life stages were tested with rabbit antisera specific to either HG-ENG-1 or HG-ENG-2 (Smant et al. 1998; Wang et al. 1999). Adaptations of the immunolabeling procedure mainly involved the fixation in 2% paraformaldehyde in M9 buffer: eggs were fixed for 5 days at 5°C; parasitic J2, J3, and male J4 were fixed for 1 day at 22°C; female J4 and adult females were fixed for 3 days at 5°C. The nematodes were cut into sections immediately after the formaldehyde fixation. J4 and adults were cut individually. Eggs, J2, and J3 were cut in bulk with a vibrating razor blade. Antibody incubations were performed at 22°C in microcentrifuge tubes mounted on a rotator. The antibodies were diluted in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 1% blocking reagent (Boehringer Mannheim, Indianapolis, IN). Rabbit antisera to HG-ENG-1 and HG-ENG-2 were tested in a 1:200 dilution, and incubations were for at least 3 h at room temperature. The primary antibodies were detected with goat anti-rabbit antibodies conjugated with either Texas Red (Jackson Immuno Research, West Grove, PA) or Alexa 488 (Molecular Probes, Eugene, OR). Incubations were with 4 to 20 µg of secondary antibody per ml in PBS, 0.1% Tween 20, 1% blocking reagent, for at least 3 h at room temperature. Labeled nematodes were taken up in a 1:1 mixture of M9 buffer and Citifluor Mountant Media #2 (Ted Pella, Redding, CA), and examined with a Zeiss Axiovert inverted epifluorescence microscope, with either an FITC filter set (Type 41001; Chroma Technology, Brattleboro, VT) or a triple dye filter set (type XF67; Omega Optical, Brattleboro, VT).

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